MEASUREMENT OF F_2-ISOPROSTANES AS AN INDEX OF OXIDATIVE STRESS IN VIVO

L. JACKSON ROBERTS, II and JASON D. MORROW
Departments of Pharmacology and Medicine, Vanderbilt University, Nashville, TN, USA

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Abstract—In 1990 we discovered the formation of prostaglandin F_2-like compounds, F_2-isoprostanes (F_2-IsoPs), in vivo by nonenzymatic free radical–induced peroxidation of arachidonic acid. F_2-IsoPs are initially formed esterified to phospholipids and then released in free form. There are several favorable attributes that make measurement of F_2-IsoPs attractive as a reliable indicator of oxidative stress in vivo: (i) F_2-IsoPs are specific products of lipid peroxidation; (ii) they are stable compounds; (iii) levels are present in detectable quantities in all normal biological fluids and tissues, allowing the definition of a normal range; (iv) their formation increases dramatically in vivo in a number of animal models of oxidant injury; (v) their formation is modulated by antioxidant status; and (vi) their levels are not effected by lipid content of the diet. Measurement of F_2-IsoPs in plasma can be utilized to assess total endogenous production of F_2-IsoPs whereas measurement of levels esterified in phospholipids can be used to determine the extent of lipid peroxidation in target sites of interest. Recently, we developed an assay for a urinary metabolite of F_2-IsoPs, which should provide a valuable noninvasive integrated approach to assess total endogenous production of F_2-IsoPs in large clinical studies. © 2000 Elsevier Science Inc.

Keywords—Free radical, Isoprostanes, Lipid peroxidation, Oxidant stress

BIOCHEMISTRY OF THE FORMATION OF ISOPROSTANES

One of the greatest needs in the field of free radical research has been the availability of a reliable noninvasive approach to assess oxidative stress status in humans [1]. It has long been recognized that methods previously developed for this purpose lack specificity, sensitivity, or are too invasive for human investigation [2]. In 1990, we reported the discovery of the formation of prostaglandin F_2-like compounds in vivo in humans by nonenzymatic free radical–induced peroxidation of arachidonic acid [3]. The mechanism by which these compounds are formed is depicted in Fig. 1. Initially, three initial arachidonoyl radicals are formed which then undergo endocyclization to form four prostaglandin H_2-like bicyclic endoperoxide intermediate regioisomers which are reduced to four F-ring regioisomers. Each regioisomer is comprised of eight racemic diastereomers. Because these compounds are isomeric to prostaglandin $F_{2\alpha}$ formed by the cyclooxygenase, they have been termed F_2-isoprostanes (F_2-IsoPs). The regioisomers are designated as different series according to the carbon number on which the side chain hydroxyl is located. This is in based on the nomenclature system for IsoPs approved by the Eicosanoid Nomenclature Committee, sanctioned by the Joint Commission on Biochemical Nomenclature (JCBN) of the International Union of Pure and Applied Chemistry (IUPAC) [4]. The vast majority of arachidonic acid is

Address correspondence to: L. Jackson Roberts, II, MD, Department of Pharmacology, 522 MRB-1, Vanderbilt University, Nashville, TN 37232, USA; Tel: (615) 343-1816; Fax: (615) 343-9446; E-Mail: jack.roberts@mcmail.vanderbilt.edu

L. Jackson Roberts, II, received his M.D. degree from the University of Iowa and was elected to Alpha Omega Alpha. He did an Internal Medicine residency at Washington University, a fellowship in Clinical Pharmacology at Vanderbilt University, and was a recipient of a Burroughs Wellcome Scholar in Clinical Pharmacology Award. He is currently Professor of Pharmacology and Medicine at Vanderbilt University.

Jason D. Morrow received his M.D. degree from Washington University. He did an Internal Medicine residency at Vanderbilt University, an Infectious Disease fellowship at Washington University, and a Clinical Pharmacology fellowship at Vanderbilt University. He is currently a Professor of Medicine and Pharmacology at Vanderbilt University. He was a Howard Hughes Medical Institute Physician Research Fellow and the recipient of an NIH Physician Scientist Award, a International Life Sciences Institute Career Development Award, and a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

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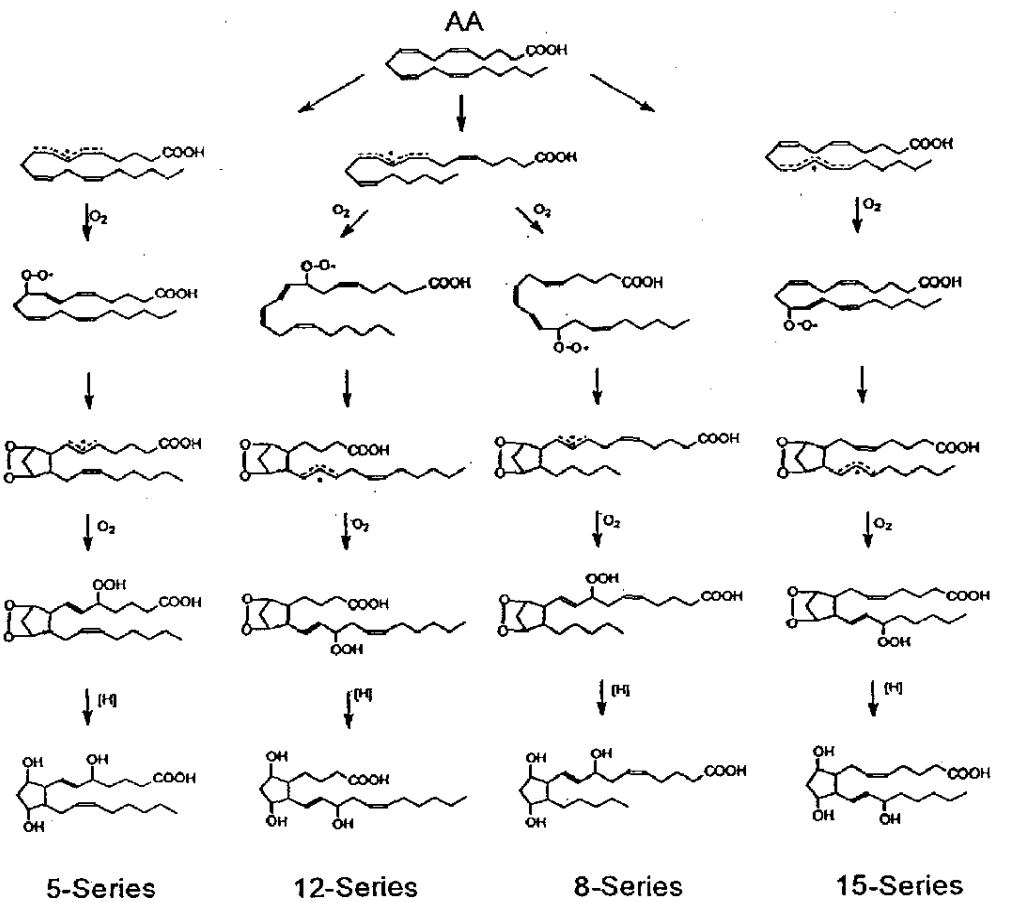

Fig. 1. Mechanism of formation of F_2-IsoPs.

present esterified in phospholipids rather than in free form. In this regard, we have found that F_2-IsoPs are initially formed esterified on phospholipids and then released in free form by a phospholipase(s) [5].

Central in the pathway of formation of IsoPs are the endoperoxide intermediates which are reduced to F-ring IsoPs. We recently demonstrated that glutathione is an important effector of this reduction [6]. However, we have shown that this reduction is not completely efficient and that the H_2-IsoP endoperoxides rearrange in vivo to form E-ring, D-ring, thromboxane-ring, A-ring, and J-ring IsoPs [7–9]. More recently, we have shown that they also undergo rearrangement to form highly reactive acyclic γ-ketoaldehydes, termed *isolevuglandins* [10]. In addition, several IsoPs have been found to exert potent and interesting bioactivity. This involves both receptor-mediated actions, e.g., vasoconstriction, in the case of F_2-IsoPs and E_2-IsoPs and biological effects due to inherent chemical reactivity, e.g., adduct formation, in the case of A_2-IsoPs, J_2-IsoPs, and isolevuglandins [9–12]. While the products of the IsoP pathway produced in addition to the F_2-IsoPs are of interest, they are not as stable as F_2-IsoPs and thus are not ideal candidates for measurement as a marker of lipid peroxidation. For this forum, therefore, we will focus on discussion of the utility of measuring F_2-IsoPs as an index of oxidative stress status.

METHODOLOGY FOR MEASUREMENT OF F_2-ISOPROSTANES

Some discussion about the methodology used for measurement of F_2-IsoPs is important. The initial discovery of F_2-IsoPs was made possible by the use of mass spectrometric analysis. We, and others, continue to use stable isotope dilution gas chromatography negative ion chemical ionization mass spectrometry for measurement of F_2-IsoPs [13]. Although mass spectrometric methodology is expensive and time consuming, it is highly specific and sensitive. The accuracy of our method of assay

Table 1. Favorable Characteristics of Measurement of F_2-IsoPs as an Index Oxidative Stress Status In Vivo

Stable compounds
Specific products of lipid peroxidation
Present in detectable quantities in all normal biological tissues and fluids, thus allowing the definition of a normal range
Levels increase substantially in animal models of oxidant injury
Levels are modulated by antioxidant status
Levels are unaffected by lipid content in the diet

is 96% and the precision is ± 5%. Enzyme-linked immunosorbent assay (ELISA) kits are now available for measurement of F_2-IsoPs by several commercial vendors. However, immunoassays for F_2-IsoPs are associated with the same potential shortcomings that have been recognized with immunoassays for prostaglandins for over 3 decades [14]. These problems are primarily related to substances in biological fluids that interfere with the immunoassay. In this regard, the immunoassays can usually be demonstrated to work very well in buffer systems that do not contain large amounts of biological substances but in complex biological fluids and tissues, interfering substances are frequently encountered. More often than not, biological samples must be purified to some extent before performing the immunoassay. Simple partial purification procedures such as extraction may actually concentrate interfering substances, thus requiring more extensive purification by thin-layer chromatography or high-performance liquid chromatography. A recent article by Proudfoot and colleagues compared measurement of F_2-IsoPs in urine by ELISA and mass spectrometry and found considerable inconsistencies [15]. Thus, at present, measurement of F_2-IsoPs by mass spectrometry remains the method of choice.

FAVORABLE CHARACTERISTICS OF F_2-ISOPROSTANES AS A MEASURE OF OXIDATIVE STRESS IN VIVO

The discovery of the formation of F_2-IsoPs was initially of biochemical interest but evolving from subsequent studies is the notion that measurement of these compounds likely represents one of the most reliable approaches to assess oxidative stress status in vivo. There are a number of favorable attributes that imply that measurement of F_2-IsoPs may provide a reliable marker of lipid peroxidation in vivo (Table 1). First, these are stable compounds. F_2-IsoPs are also specific products of free radical–induced lipid peroxidation. F_2-IsoPs have been found to be present in detectable quantities esterified in all normal biological tissues and in free form in all normal biological fluids. This is important because it allows the definition of a normal range such that small increases in their formation can be detected in situations of mild oxidant stress. It should be mentioned that IsoPs are not a major product of lipid peroxidation vide infra but levels are present in vivo that can be readily detected by currently available methodology. Importantly, the formation of F_2-IsoPs has been shown to increase dramatically in well-established animal models of oxidant injury, e.g., administration of CCl_4 to normal rats and administration of diquat to Se-deficient rats [3]. Further, levels are modulated by endogenous antioxidant status, e.g., vitamin E and Se, and by exogenous administration of antioxidant agents [16–18]. Finally, there may be a concern that levels of F_2-IsoPs may be influenced by lipid content in the diet which can contain IsoPs as a result of oxidation of dietary arachidonic acid. However, we had previously shown urinary levels of F_2-IsoPs in subjects ingesting a normal diet were unchanged after 4 d of a diet consisting of only glucose polymers [3]. In a recent study by Richelle and colleagues, it was also confirmed that levels of IsoPs are unaffected by lipid content of the diet [19].

DIRECT COMPARISON OF MEASURING F_2-ISOPS WITH OTHER MEASURES OF LIPID PEROXIDATION TO ASSESS OXIDANT INJURY IN VITRO AND IN VIVO

To explore whether the value of measuring F_2-IsoPs surpasses that of some routinely used measures of lipid peroxidation in vivo, we directly compared measurements of F_2-IsoPs with malondialdehyde (MDA) and lipid hydroperoxides, commonly used measures of lipid peroxidation, both in vitro and in vivo. The results obtained were most revealing.

Comparison between the formation of malondialdehyde and F_2-IsoPs

Initially, we compared the time-course of formation of F_2-IsoPs with MDA in vitro during oxidation of rat liver microsomes with iron/ADP/ascorbate [20]. MDA was measured by the thiobarbituric acid reacting substances (TBARS) assay [21]. As shown in Fig. 2A, the time-course of formation of both MDA and F_2-IsoPs were highly correlated. Of note, however, are the different scales on the two y-axes indicating that there was approximately 25,000 times more MDA generated than F_2-IsoPs. We then compared the amounts of MDA and esterified F_2-IsoPs formed in the liver of rats after ad-

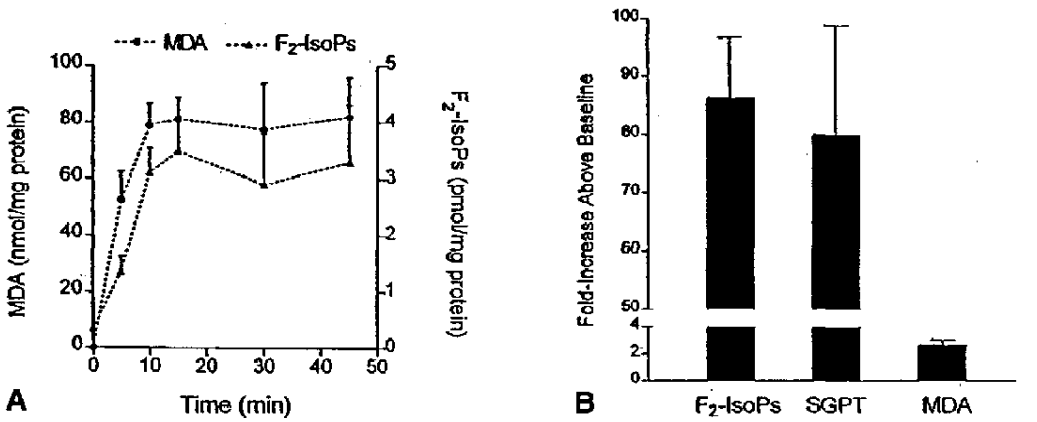

Fig. 2. (A) Time-course of formation of MDA and F_2-IsoPs during oxidation of rat liver microsomes. (B) Levels of F_2-IsoPs esterified in liver lipids, levels of MDA in liver, and plasma concentrations of SGPT (serum glutamic pyruvic transaminase) following administration of CCl_4 to rats.

ministration of CCl_4 to induce a severe oxidant injury to the liver (Fig. 2B). As a index of the severity of liver injury, plasma concentrations of serum glutamic pyruvic transaminase (SGPT) were also measured. Levels of esterified F_2-IsoPs in the liver increased strikingly by approximately 85-fold. This was accompanied by a comparable increase in plasma concentrations of SGPT, indicating a good correlation between the magnitude F_2-IsoP production and severity of hepatocellular injury. In contrast, levels of MDA increased only less than 3-fold. Thus, whereas the relative increases in the formation of MDA and F_2-IsoPs were found to correlate highly during oxidation of microsomal lipids in vitro, the relative increase in levels of F_2-IsoPs detected in the setting of CCl_4-induced oxidant injury in vivo far surpassed the increase in levels of MDA detected. The reason why the relative increase in MDA after administration of CCl_4 in vivo was much less compared with the increase in F_2-IsoPs remains speculative but may be due to rapid metabolic clearance [22]. This demonstration of the greater utility of measurement of F_2-IsoPs compared with MDA in vivo as an index of oxidant injury is even further enhanced by the recognition that the TBARS assay is not specific for MDA nor is MDA a specific marker of lipid peroxidation [2,23].

Comparison between the formation of lipid hydroperoxides and F_2-IsoPs

We also carried out separate studies comparing the generation of lipid hydroperoxides with F_2-IsoPs both in vitro and in vivo. In collaboration with Frei and colleagues [24], we compared the formation of cholesterol ester lipid hydroperoxides and F_2-IsoPs during Cu^{+2}-induced oxidation of human low-density lipoprotein (LDL). As shown in Fig. 3A, the relative increases and time-courses of formation of cholesterol ester lipid hydroperoxides, measured by chemiluminescence assay, and F_2-IsoPs were highly correlated. Of note again, however, is that the levels of cholesterol ester lipid hydroperoxides are plotted on a micromole scale whereas the levels of F_2-IsoPs are plotted as nanomoles, indicating that approximately 1000-times higher amounts of cholesterol ester lipid hydroperoxides were formed compared with F_2-IsoPs. The decline in levels of both during later times of incubation of LDL with Cu^{+2} were due to hydrolysis of the lipid hydroperoxides and F_2-IsoPs. Although not shown, this was confirmed by demonstrating that the decline in levels of esterified F_2-IsoPs was mirrored by an increase in levels of free F_2-IsoPs in the incubation buffer.

In collaboration with Matthews and colleagues [22], we also undertook studies in which we compared the relative amounts of formation of arachidonic acid–derived lipid hydroperoxides and F_2-IsoPs in vivo esterified in liver lipids and free in the circulation during administration of CCl_4 to rats [17]. In these experiments, lipid hydroperoxides were measured by a highly sensitive and accurate stable isotope dilution negative ion chemical ionization gas chromatography/mass spectrometry (GC/MS) method reduction of the lipid hydroperoxides to stable alcohols (hydroxyeicoastetraenoic acids [HETEs]). As shown in Fig. 3B, the fold-increase in levels of F_2-IsoPs esterified in liver of rats treated with CCl_4 compared to levels measured in untreated rats greatly exceeded that of HETEs. Also of note is that F_2-IsoPs could be detected in the circulation of untreated rats and levels increased more than 20-fold following administration of CCl_4, whereas HETEs could not be detected in the circulation, even in this setting of severe

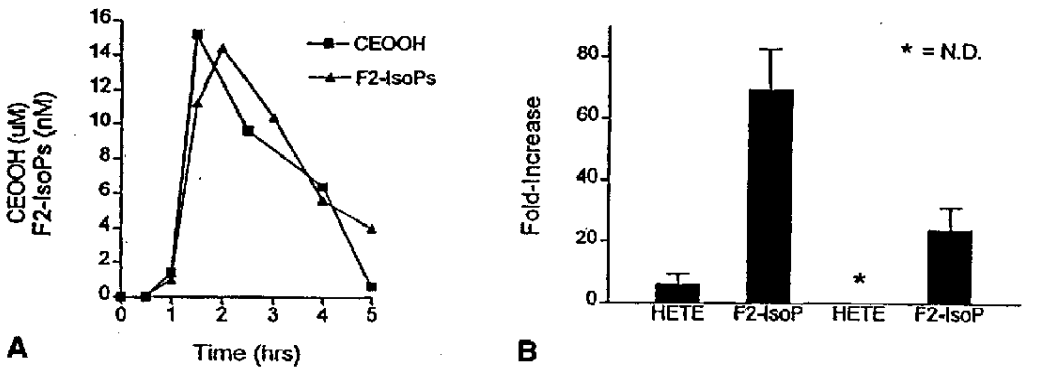

Fig. 3. (A) Time-course of formation of cholesterol ester lipid hydroperoxides and esterified F_2-IsoPs during Cu^{+2}-induced oxidation of LDL. (B) Levels of lipid hydroperoxides and F_2-IsoPs esterified in liver and circulating concentrations in plasma following administration of CCl_4 to rats. Lipid hydroperoxides were measured following reduction to alcohols (HETE's). N.D. = not detected.

CCl_4-induced oxidant injury using a highly sensitive method of assay. The results of this study again highlights the greater utility of measuring F_2-IsoPs as an index of free radical–induced lipid peroxidation compared with another measure of lipid peroxidation, namely measurement of lipid hydroperoxides.

VARIOUS APPROACHES TO ASSESS ENDOGENOUS PRODUCTION OF F_2-ISOPS

There are several approaches that can be utilized to assess endogenous production of F_2-IsoPs, each of which has certain advantages and/or drawbacks.

Measurement of free unmetabolized IsoPs

Sampling of biological fluids for measurement of free unmetabolized F_2-IsoPs usually involves plasma or urine although other biological fluids can also be used in special situations, e.g., cerebrospinal fluid. Plasma sampling has the potential problem of artifactual generation of IsoPs by autoxidation of plasma arachidonic acid if the sample is not processed and stored properly. Plasma samples cannot be stored at −20°C because autoxidation can occur during storage at this temperature [25]. However, we have found that plasma can be stored at −70°C for at least 6 months without the occurrence of generation of F_2-IsoPs by autoxidation. Autoxidation is not a problem with urine [3], which is understandable because urine does not have a high lipid content. Conceptually, concentrations of F_2-IsoPs in plasma can provide a useful index of total endogenous production of IsoPs because levels in plasma presumably derive from all tissues in the body. A potential contribution of local formation of F_2-IsoPs in the kidney may confound interpretation of urinary levels of unmetabolized F_2-IsoPs [11]. As mentioned, IsoPs are initially formed esterified in lipids and then released in free form. This can be a dynamic process [4]. Depending on the experimental situation, timing of sampling of blood for determination of F_2-IsoPs may not be critical. Because the elimination half-life of IsoPs in the circulation is relatively short, less than 20 min [26], measurement of F_2-IsoPs in a single sample of blood only provides an index of IsoP formation during a relatively short period of time. In chronic disease states in which there may be a relatively steady rate of formation and release of IsoPs from phospholipids into the circulation, timing of sampling of blood is not critical. However, in some chronic disease states there may be significant intraday fluctuations in the formation of IsoPs. In dynamic situations in which there is an oxidant insult for only a relatively short period of time, e.g., ischemia/reperfusion injury, multiple sequential sampling of blood is necessary to assess the full magnitude of the increase in IsoP generation during rapidly changing rates of production over time [16].

Measurement of esterified F_2-IsoPs

Because IsoPs are initially formed esterified to phospholipids, this can be utilized to assess overproduction of F_2-IsoPs in specific target sites of interest. With this approach, phospholipids and neutral lipids are subjected to Folch lipid extraction and then hydrolyzed with base after which liberated free F_2-IsoPs are quantified [13]. Although sampling of tissues for analysis is primarily limited to studies in experimental animals, the sensitivity of the mass spectrometric assay is sufficient to allow determination of levels of F_2-IsoPs in small biopsy specimens from human tissues. This approach has been utilized to explore levels of esterified F_2-IsoPs in postmortem samples obtained from humans, e.g., normal and

atherosclerotic vessels in which significantly increased levels of F_2-IsoPs were found in atherosclerotic plaques compared with normal vessels [27,28]. However, precautions such as rapid procurement of tissues after death and immediate storage at −70°C must be taken to avoid artifactual generation of IsoPs by autoxidation. The other area where we and others have utilized this approach rather extensively in living subjects is to assess levels of F_2-IsoPs esterified in plasma lipoproteins in studies exploring the LDL oxidation hypothesis of atherogenesis [16,29]. In this regard, levels of F_2-IsoPs esterified in plasma lipoproteins have been found to be elevated in patients with hypercholesterolemia and patients who smoke [16,29,30], conditions that are high risk factors for atheroslerosis. In an equal volume of plasma, F_2-IsoPs esterified in plasma lipoproteins are present at levels approximately 4-fold higher than levels of free F_2-IsoPs. Thus, for such studies, only small amounts of plasma need to be obtained for analysis (~0.5 ml).

Measurement of a urinary metabolite of F_2-IsoPs

Obtaining plasma for determination of F_2-IsoPs, although only minimally invasive, is frequently not suitable for large clinical trials because of the logistics of drawing blood and the requirement that the plasma be rapidly isolated and stored at −70°C. Although collection of urine is feasible in large clinical trials, the interpretation of measurement of unmetabolized F_2-IsoPs in urine as an index of total endogenous IsoP production can be confounded by the potential contribution of local IsoP production in the kidney. Therefore, we recently carried out a study to identify a urinary metabolite of an F_2-IsoP to circumvent these problems. It has been well established in the prostaglandin field that measurement of the urinary excretion of metabolites of prostaglandins represents the most reliable approach to assess total endogenous production of prostanoids [31]. Thus, measurement of the urinary excretion of an F_2-IsoP metabolite should also afford an accurate measure of endogenous production of IsoPs. This has the additional advantage in that blood does not have to be obtained and also measurement of the level of a metabolite in urine collected over many hours can provide an integrated index of IsoP production over time.

One of the F_2-IsoPs, which we have shown is produced in vivo, is 15-F_{2t}-IsoP (8-iso-prostaglandin $F_{2\alpha}$) [32]. Metabolism of prostaglandins in most instances has been found to produce a plethora of metabolites. However, we found that a single metabolite predominated in the profile of derivatives produced from metabolism of 15-F_{2t}-IsoP. This metabolite was identified by mass spectrometric analysis as 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP [33] (Fig. 4). This metabolite was synthesized and converted to an [$^{18}O_4$] derivative for use as an internal standard and recently we developed a stable isotope dilution negative ion chemical ionization GC/MS method for its analysis [34]. Levels of this metabolite in normal individuals were found to be 0.39 ± 0.18 ng/mg creatinine (mean ± 2 SD). The levels of this metabolite increased a mean of 24-fold compared with baseline in urine collected over 12 h after administration of CCl_4 to rats. Additionally, the excretion of this metabolite was found to be increased a mean of 2.5-fold in patients with polygenic hypercholesterolemia and these increases were suppressed by a mean of 54% following 8 weeks of treatment with a combination of vitamin E, vitamin C, and β carotene. These data suggest that quantification of the urinary excretion of 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP will contribute importantly to our ability to reliably assess free radical–induced lipid peroxidation in vivo and provide an approach that should be applicable to large clinical studies. Development of an ELISA assay for this metabolite is currently under commercial development. Assuming that the accuracy of the ELISA assay for the metabolite in urine can be validated by GC/MS, this may eventuate in the wide availability of this measure of lipid peroxidation by investigators in the free radial field.

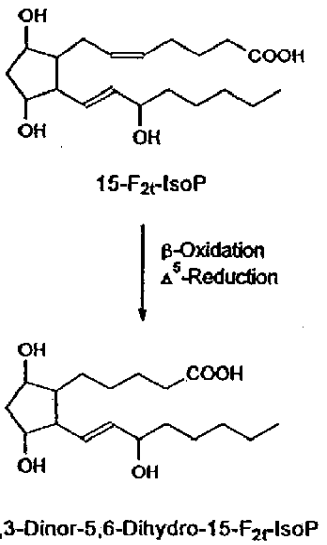

Fig. 4. Formation of the major urinary metabolite of 15-F_{2t}-IsoP by processes of one step of β-oxidation and reduction of the Δ^5-double bond.

SUMMARY AND CONCLUSIONS

In summary, initially the discovery of F_2-IsoPs was primarily of biochemical interest. This discovery, how-

Table 2. Disorders in Which Measurements of F_2-IsoPs Has Implicated a Role for Free Radicals in the Disease Process

Smoking [30,35]	Rhabdomyolysis renal injury [50,51]
Alzheimer's disease [36–38]	Acute cholestasis [52,53]
Huntington's disease [39]	Adult respiratory distress syndrome [54]
Hypercholesterolemia and atherosclerosis [16,27–30,40]	Halothane hepatoxicity [55]
Hyperhomocysteinemia [41]	Acetaminophen poisoning [11]
Hepatorenal syndrome [42]	Ischemia/reperfusion injury [16,56–59]
Scleroderma [43]	Cr (IV) poisoning [60]
Age-related decline in renal function [44]	Diquat poisoning [3,61]
Se deficiency [18]	Cisplatin-induced renal dysfunction [62]
Vitamin E deficiency [18]	Transplant organ injury during cold preservation [63]
Retinopathy of prematurity [45]	Chronic obstructive lung disease [64]
Alcohol-induced liver injury [46,47]	Interstitial lung disease [65]
Allergic Asthma [48]	Organophosphate poisoning [66]
Diabetes [49]	CCl_4-induced hepatotoxicity [3,26]

ever, has evolved over the last several years in a number of areas. These include the discovery of a number of additional classes of compounds that are generated as products of the IsoP pathway and the findings that several of these compounds can exert potent biological activity either through receptor-mediated actions or in the case of A_2/J_2-IsoPs and isolevuglandins because of their inherent chemical reactivity. However, an important aspect of the discovery of IsoPs is that evidence continues to accumulate suggesting that measurement of F_2-IsoPs represents one of the most reliable approaches to assess oxidative stress status in vivo. Utilizing measurements of F_2-IsoPs has implicated strongly, often for the first time, a role for free radicals in the pathogenesis of a large number of diseases (Table 2). In most of the studies listed in Table 2, F_2-IsoPs were measured by mass spectrometry which is accurate but requires expensive instrumentation. In this regard, the major limitation for the wide-spread use of measurements of F_2-IsoPs by investigators in free radical research has been the issue of reliability and accuracy of immunoassays for F_2-IsoPs. Hopefully, these potential problems regarding the accuracy of immunoassays for F_2-IsoPs can be overcome in the future which would undoubtedly lead to a great expansion in the use of measurement of F_2-IsoPs to assess oxidative stress status in vivo.

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ABBREVIATIONS

F_2-IsoPs—F_2-isoprostanes
MDA—malondialdehyde
SGPT—serum glutamic pyruvic transaminase
TBARS—thiobarbituric acid reacting substances
LDL—low-density lipoprotein
HETE—hydroxyeicosatetraenoic acid